ÖGH Herpetozoa
Austrian Herpetological Society

Herpetozoa 38: 43–60 (2025)
DOI 10.3897/herpetozoa.38.e146004

A new endemic karst-associated species of lance-headed pit viper (Squamata, Viperidae, *Protobothrops*) from Laos

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Academic editor: Peter Mikulíček ♦ **Received** 5 January 2025 ♦ **Accepted** 10 February 2025 ♦ **Published** 25 February 2025

Abstract

We describe a new species of lance-headed pit viper from north-western Laos, based on morphological and molecular (6092 bp from cyt *b*, ND4, COI, 12S rRNA and 16S rRNA mitochondrial DNA genes and c-mos and RAG1 nuclear DNA genes) lines of evidence. *Protobothrops flavirostris* **sp. nov.** is easily distinguished from its congeners by the following combination of morphological characters: dorsal scales in 23–21–17 rows, all keeled; ventral scales 215; subcaudal scales 79, all paired; supralabials 7–8; infralabials 10; horn-like projections on supraoculars absent; head triangular with a typical lance-shaped pattern on its dorsal surface; three faint dark vertical stripes on the snout; head blackish-brown with rostral, nasals, preoculars, loreals and the two anterior supralabials, as well as the anterior parts of supraoculars yellow-orange; dorsal surfaces of body and tail brown or greyish-brown, dorsum with large dark reddish-brown cross-shaped blotches, edged in black, somewhat fused together forming an interrupted zigzag line and a row of large brown ventrolateral blotches on each side. The new species differs from the morphologically similar species *Protobothrops kelomohy* by a significant divergence in cytochrome *b* mitochondrial DNA gene sequences (p = 7.8%). The new species is currently known only from tropical limestone forest of Vientiane Province, north-western Laos (elevation 362 m a.s.l.). We suggest the new species be considered as Endangered (EN) following the IUCN's Red List categories.

Key Words

Indochina, limestone, morphology, molecular phylogeny, *Protobothrops flavirostris* sp. nov., systematics, Vientiane Province

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Introduction

The Asian lance-headed pit vipers of the genus *Protobothrops* Hoge & Romano-Hoge, 1983, comprise terrestrial venomous snakes that are widely distributed across South, Southeast and East Asia (Uetz et al. 2025). Most *Protobothrops* are highly venomous, making this genus an important study object due to their medical significance and potential impacts on human health through venomous snakebites. However, the taxonomy of the genus *Protobothrops* remains controversial, especially the widely distributed species complexes, such as *P. jerdonii* (Günther) and *P. mucrosquamatus* (Cantor) (Guo et al. 2016, 2019). Several recent studies on the taxonomy and molecular phylogeny of *Protobothrops* have demonstrated that the diversity of the genus is underestimated, with several new species described in recent years (e.g. Huang et al. (2012); Pan et al. (2013); Sumontha et al. (2020)).

The genus *Protobothrops* is morphologically characterised by the presence of a well-developed loreal pit; a large nasal pore located close to the external rim of the nostril; by relatively short and forked hemipenes with spinulose proximal parts and calyculate distal parts; by elongate or even slender body shape and slender and tapering tail; by head clearly distinct from the thin neck; by flattened head and snout; by dorsal head scales uniformly small, except the internasals and supraoculars which are relatively enlarged; by having comparatively small eyes with vertically elliptic pupils; by the first supralabial being always separated from the nasal by a complete suture; by second supralabial bordering the loreal pit; by presence of a single, elongated subocular; by dorsal scales distinctly keeled; a generally high number of ventrals, at least 160, but more than 185 in most species; by all subcaudals paired; cloacal plate entire; background colouration in shades of grey or brown, never uniformly green; by having a variable dorsal pattern, usually consisting of dark blotches on each side of the dorsum, more or less fused forming an irregular zigzag pattern (following David et al. (2023)). Currently, the genus *Protobothrops* comprises 15 nominal species, namely: *P. cornutus* (Smith), *P. dabieshanensis* Huang, Pan, Han, Zhang, Hou, Yu, Zheng & Zhang, *P. elegans* (Gray), *P. flavoviridis* (Hallowell), *P. himalayanus* Pan, Chettri, Yang, Jiang, Wang, Zhang & Vogel, *P. jerdonii*, *P. kaulbacki* (Smith), *P. kelomohy* Sumontha, Vasaruchapong, Chomngam, Suntrarachun, Pawangkhanant, Sompan, Smits, Kunya & Chanhome, *P. mangshanensis* (Zhao), *P. maolanensis* Yang, Orlov & Wang, *P. mucrosquamatus*, *P. sieversorum* (Ziegler, Herrmann, David, Orlov & Pauwels), *P. tokarensis* (Nagai), *P. trungkhanhensis* Orlov, Ryabov & Nguyen and *P. xiangchengensis* (Zhao, Jiang & Huang). The taxonomic status of several species within the genus remains controversial: *P. sieversorum* was originally assigned to a distinct genus *Triceratolepidophis* Ziegler, Herrmann, David, Orlov & Pauwels, while *P. mangshanensis* was classified as a member of a monotypic genus *Zhaoermia* Gumprecht & Tillack; currently, based on the phylogenetic data, both names are considered as junior synonyms of *Protobothrops* and no subgenera within the latter are recognised (see Guo et al. (2016); David et al. (2023)).

Presently, seven recognised *Protobothrops* species are recorded from the Indochinese Region, which includes Laos, Cambodia, Vietnam and Thailand (see Fig. 1); namely, *P. cornutus*, *P. jerdonii*, *P. kelomohy*, *P. maolanensis*, *P. mucrosquamatus*, *P. sieversorum* and *P. trungkhanhensis* (David et al. 2023; Poyarkov et al. 2023). During our recent field surveys in the limestone karst forests of Vang Vieng District, Vientiane Province, in north-western Laos, we encountered a single snake specimen provisionally assigned to the genus *Protobothrops*, based on its external morphology. Subsequent phylogenetic analyses, based on five mitochondrial DNA (hereafter, mtDNA) genes (cyt *b*, ND4, COI, 12S rRNA and 16S rRNA) and two nuclear DNA (hereafter nDNA) genes (c-mos and RAG1), confirmed its placement within the genus *Protobothrops* and suggested that the Vang Vieng specimen represents a deeply divergent lineage, sister to the recently-described *P. kelomohy* from north-western Thailand. Closer morphological examination of scalation, colouration, pattern and morphometric characteristics of the Vang Vieng specimen demonstrated clear morphological differences from its sister species as well as from all other species of the genus *Protobothrops*. By integration of morphological and molecular data, we describe below the Vang Vieng specimen of lance-headed pit vipers from Laos as a new species.

Material and methods

Sampling

In October 2024, we conducted fieldwork along the base of limestone karst formations and within caves in Vang Vieng District, Vientiane Province, Laos (Fig. 1). We recorded geographic coordinates and elevations using GAIA GPS Version: v.2024.7.1 in the WGS 84 datum. The specimen was collected by means of snake hooks and a secure plastic tub, photographed in life and euthanized using MS-222 solution 5 days after capture. The specimen was fixed in 4% buffered formalin for 24 h and later stored in 70% ethanol. The specimen was subsequently deposited in the collection of the National University of Laos (NUOL), Vientiane Capital, Laos. Prior to specimen preservation, we extracted tissues for genetic analyses from the liver and heart and stored them in 96% ethanol.

Molecular laboratory methods

We extracted total genomic DNA from ethanol-preserved muscle tissue using standard phenol-chloroform extraction procedures (Sambrook et al. 1989), followed by isopropanol precipitation. We used polymerase chain

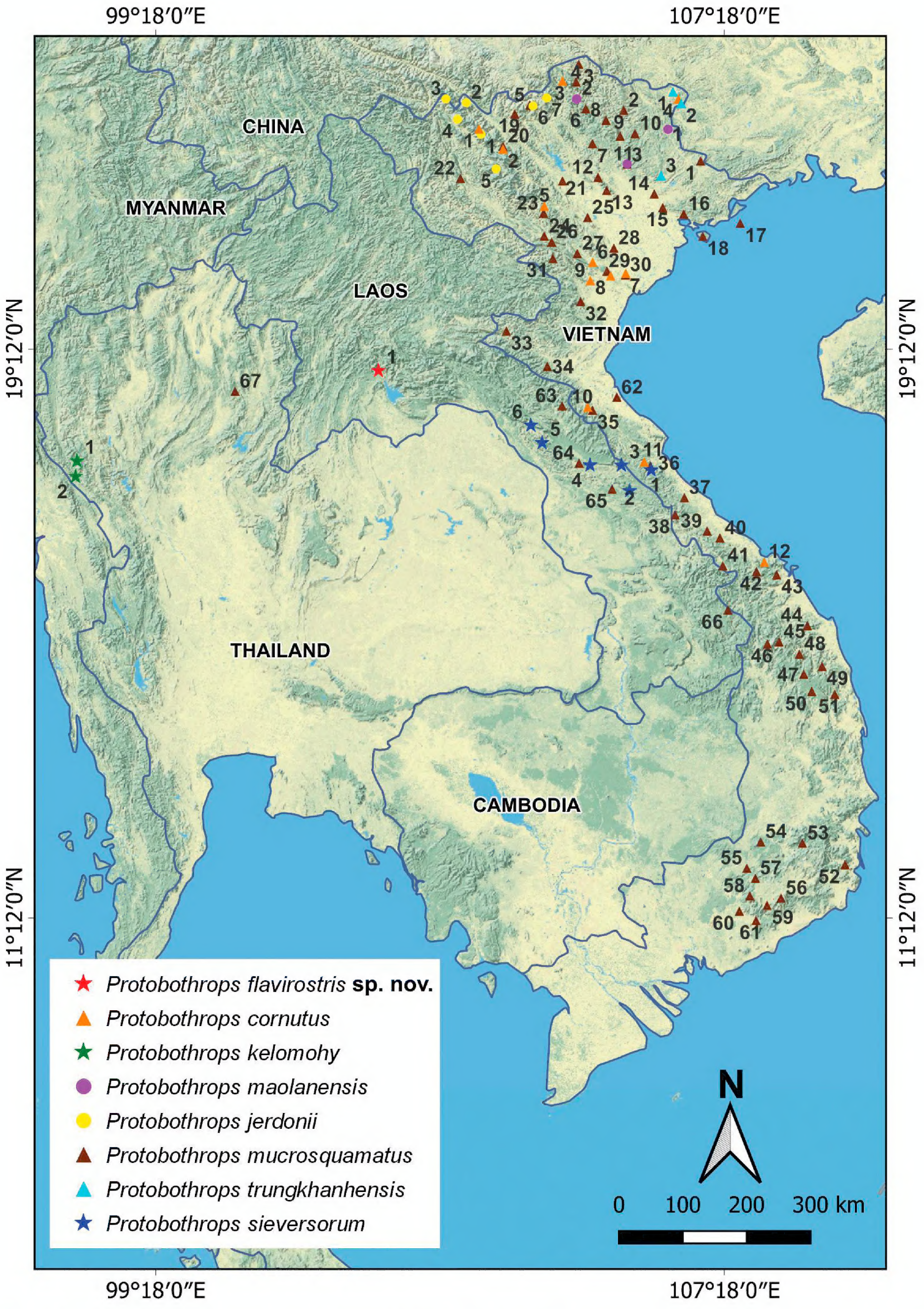

Figure 1. Distribution of the members of the genus *Protobothrops* in Indochina. Numbers indicate different localities where the species have been recorded (localities are numbered for each species independently; see Suppl. material 1: appendix 6 for the details on locality information and data sources). Symbols indicating *Protobothrops* species are identical to those used in Fig. 2.

reaction (PCR) to amplify five mtDNA fragments: complete sequences of cytochrome *b* (cyt *b*), NADH dehydrogenase subunit 4 (ND4) and cytochrome *c* oxidase subunit I (COI), as well as fragments of 12S rRNA and 16S rRNA genes. We also amplified the fragments of two nDNA genes, including the complete sequence of the oocyte maturation factor (c-mos) and the first fragment of the recombination activating gene 1 (RAG1). The primers used for both PCR and sequencing are summarised in Suppl. material 1: appendix 1.

For cyt *b*, ND4, COI, 12S and 16S rRNA, c-mos and RAG1 gene fragments, we used the modified PCR protocols of Dahn et al. (2018), Salvi et al. (2013), Nagy et al. (2012), Green et al. (2010), Slowinski and Lawson (2002) and Groth and Barrowclough (1999), respectively; all details on PCR conditions are summarised in Suppl. material 1: appendix 2. We ran all amplifications using an iCycler Thermal Cycler (Bio-Rad). We loaded the PCR products on to 1% agarose gels in the presence of ethidium bromide and visualised them by electrophoresis. The successful targeted PCR products were purified by the Diatom DNA PCR Clean-Up kit and outsourced to Evrogen® (Moscow, Russia) for sequencing; sequence data collection and visualisation were performed on an ABI 3730xl Automated Sequencer (Applied Biosystems).

Molecular phylogeny

To estimate the phylogenetic relationships of the genus *Protobothrops*, we used the newly-obtained cyt *b*, ND4, COI, 12S rRNA, 16S rRNA, c-mos and RAG1 sequences together with previously-published sequences of these genes for the genus *Protobothrops*. Six representatives of the subfamily Crotalinae Oppel (genera *Crotalus* Linnaeus, *Agkistrodon* Palisot de Beauvois, *Deinagkistrodon* Gloyd, *Gloydius* Hoge & Romano-Hoge and *Trimeresurus* Lacépède) were used as outgroups and the sequence of *Azemiops feae* Boulenger (subfamily Azemiopinae Liem, Marx & Rabb) was used to root the tree (summarised in Suppl. material 1: appendix 3).

We first aligned the nucleotide sequences using the default parameters in MAFFT online (Katoh et al. 2019), then visually checked them in BioEdit 7.0.5.2 (Hall 1999) and adjusted them when required. The mean uncorrected genetic *p*-distances between sequences were calculated with MEGA 6.0 with the pairwise deletion option (Tamura et al. 2013), based on cyt *b* sequences of pit vipers of the genus *Protobothrops*. The best-fit substitution models for the dataset were selected for genes and codon positions using PartitionFinder 2.1.1 (Lanfear et al. 2012) with corrected Akaike Information Criterion (AICc); the data on suggested substitution models and other parameters for each gene are summarised in Suppl. material 1: appendix 4.

We first estimated matrilineal genealogical relationships between the *Protobothrops* species for the concatenated mtDNA dataset which included the fragments of cyt b, ND4, COI, 12S rRNA and 16S rRNA gene sequences. Additionally, for the two nuclear markers examined (c-mos and RAG1), we constructed allele networks using the median-joining method in PopArt ver. 1.5 (Leigh and Bryant 2015) with a 95% connection limit. For the purpose of allele network construction, sequences with more than one heterozygous site were resolved in PHASE 2.1.1 (Stephens et al. 2001) for which the input data were prepared in SeqPHASE (Flot 2010). PHASE was run under default settings, except for the probability threshold, which was set to 0.7. As no significant topological conflict was observed between the matrilineal genealogy and allele networks for nuclear genes, we further relied on the total evidence concatenated dataset, comibining the mtDNA and nDNA fragments in one alignment, also following the previous works on *Protobothrops* phylogentics (e.g. Guo et al. (2016); Li et al. (2020)).

We inferred the phylogenetic relationships of *Protobothrops* using Bayesian Inference (BI) and Maximum Likelihood (ML) approaches. We used the IQ-TREE online server (Nguyen et al. 2015) to generate the ML tree and assessed the confidence in tree topology by 10,000 ultrafast bootstrap replications (UFBS). We conducted BI in the terminal version of MrBayes 3.1.2 (Huelsenbeck and Ronquist 2001). Metropolis-coupled Markov Chain Monte Carlo (MCMCMC) analyses were run with one cold chain and three heated chains for 20 million generations and sampled every 2,000 generations. The run was checked to ensure the effective sample sizes (ESS) were all above 200 by exploring the likelihood plots using TRACER v. 1.7 (Rambaut et al. 2018). We discarded the initial 1000 trees as burn-in. For BI, we assessed the confidence in tree topology using the posterior probability (PP) of the nodes (Huelsenbeck and Ronquist 2001). We *a priori* regard the nodes with UFBS values of 95% or higher and PP values over 0.95 as strongly supported; UFBS values between 95% and 90% and PP values between 0.95 and 0.90 were regarded as well-supported and lower values were regarded as having a lack of node support (Minh et al. 2013).

Morphological examination

Measurements and meristic counts followed Idiiatullina et al. (2023, 2024a, b, c). Paired meristic characters were given in the left/right order. We used a Mitutoyo digital caliper to take the following measurements to the nearest 0.1 mm: **ED**, horizontal eye diameter; **EN**, eye to nostril (distance between anterior margin of eye and posterior margin of nostril); **IN**, internarial distance (horizontal distance between nostrils); **NL**, nostril-loreal pit distance; **HD**, maximum head depth; **HL**, head length (from the tip of rostral to the posterior end of the jaw); **HW**, maximum head width; **SnL**, snout length (from the tip of rostral to the anterior eye margin); **SVL**, snout-vent length; **TaL**, tail length; **TL**, total length. Examined meristic characters include **CP**, cloacal plate(s) (divided or single);

ASR, anterior number of dorsal scale rows (at one HL behind the head); **IL**, number of infralabial scales; **IOS**, interorbital scales, counted along a row between the middle of supraocular scales; **MSR**, number of dorsal scale rows at mid-body (at the level of the ventral plate corresponding to half of the total number of ventrals); **PosOc**, number of postocular scale(s); **PSR**, posterior number of dorsal scale rows (at one HL before the cloacal plate); **PreV**, number of preventrals (scales directly preceding the ventrals, unpaired, wider than long, but not in contact on each side with the 1st dorsal scale row); **SC**, number of subcaudal scales, not including the terminal pointed scute; **SL**, number of supralabial scales; **VEN**, number of ventral scales (counted following Dowling (1951)). Comparative data for the other species of *Protobothrops* were obtained from the literature (e.g. Maki (1931); Zhao et al. (1998); Orlov et al. (2009); Yang et al. (2011); Huang et al. (2012); Pan et al. (2013); Luu et al. (2015); Sumontha et al. (2020); David et al. (2023)).

Other abbreviations. **Dist.**: District; **Mt.**: Mountain; **NP.**: National Park; **NR.**: Nature Reserve; **Is.**: Island; **WS**: Wildlife Sanctuary; **Prov.**: Province.

Species delimitation

The general lineage concept (GLC: de Queiroz (2007)) adopted herein proposes that a species constitutes a population of organisms evolving independently from other such populations owing to a lack of gene flow. By "independently," it is meant that new mutations arising in one species cannot spread readily into another species (Barraclough et al. 2003; de Queiroz 2007). Under the GLC implemented herein, molecular phylogenies were used to recover monophyletic mitochondrial lineages of individual(s) (i.e. populations) in order to develop initial species-level hypotheses — the grouping stage of Hillis (2019). The differentiation of the lineages was further assessed by haplotype network analysis of two nuclear gene sequences (c-mos and RAG1), along with the analysis of external morphology and discrete colour pattern data used to search for unique characters and patterns and compare their consistency with the previous species-level hypotheses designations — the construction of boundaries representing the hypothesis-testing step of Hillis (2019) — thus providing lineage diagnoses independent of the molecular analyses. This approach was proposed to avoid the inherent errors of simultaneous species delimitation (phylogeny) and diagnostics (taxonomy) (Frost and Hillis 1990; Frost and Kluge 1994; Hillis 2019).

Results

Sequence variation

A total of 6092 aligned base pairs (1105 bp from cyt *b*, 861 bp from ND4, 520 bp from 12S rRNA, 1364 bp from 16S rRNA, 656 bp from COI, 584 from c-mos and 996 bp from RAG1 genes) were obtained from the five mtDNA and two nDNA fragments, respectively (see Suppl. material 1: appendix 3 for details). Protein-coding sequences were translated into amino acids to confirm that no pseudogenes had been amplified. We deposited the newly-obtained sequences in GenBank (see Suppl. material 1: appendix 3 for the accession numbers and voucher details).

Phylogenetic relationships

The matrilineal genealogy of the genus *Protobothrops* is presented in Suppl. material 1: fig. S1; the haplotype networks for two nuclear genes (c-mos and RAG1) are shown in Suppl. material 1: fig. S2. For both nuclear DNA genes examined, the newly-discovered population of *Protobothrops* sp. from Laos was characterised by unique haplotypes, separated from its closest relatives by at least two or one mutational steps (for c-mos and RAG1, respectively; see Suppl. material 1: fig. S2). The network analysis of nuclear genes indicates incomplete lineage sorting in RAG1, but not in c-mos (Suppl. material 1: fig. S2). Within the genus *Protobothrops*, most species were charcracterised by distinct nuclear gene haplotypes with the exception of two species pairs, which shared the same RAG1 haplotypes: *P. maolanensis* and *P. trungkhanhensis*, as well as *P. dabieshanensis* and *P. mucrosquamatus* (see Fig. 2B). Overall, the two nuclear genes examined demonstrated phylogenetic resolution not strong enough to assess the phylogenetic relationships amongst the species of the genus *Protobothrops*. Therefore, for subsequent analyses, we relied on the total evidence dataset, resulting from concatenation of mtDNA and nuDNA sequences in one alignment. The topology of the resulting tree (Fig. 2) was almost identical to the mtDNA matrilineal genealogy (Suppl. material 1: fig. S1) with few exceptions which are addressed below.

Phylogenetic trees obtained using ML and BI analyses of the concatenated mtDNA + nuDNA dataset were congruent apart from the topologies of several poorly-supported deeper nodes, which are irrelevant to the discussion in the present study (see Fig. 2). Both ML and BI analyses strongly confirmed the monophyly of the genus *Protobothrops* (100/1.0; hereafter, node support values are given for ML UFBS/BI PP, respectively). Our analyses recovered three major, strongly-supported clades within the genus *Protobothrops* (Fig. 2):

1. Clade A (100/1.0) includes a single species from central China — *P. mangshanensis*, which was, until recently, regarded as a member of the monotypic genus *Zhaoermia*. Clade A demonstrates a sister relationship to all remaining species of the genus *Protobothrops*, which form a moderately supported group (95/0.89) (Fig. 2).

2. Clade B (100/1.0) regroups several species from the mountains of central Indochina and the Eastern Himalayas, including *P. sieversorum* (the type species of the monotypic genus *Triceratolepidophis*), *P. himalayanus*, *P. kaulbacki*, *P. kelomohy* and the newly-discovered population of *Protobothrops* sp. from Vang Vieng, Laos. Inside this clade, *P. sieversorum* from the Annamite (or Truong Son) Mountains in eastern Indochina is the most distant, forming a sister lineage to all the remaining species (93/0.97), while the two Himalayan species (*P. himalayanus* and *P. kaulbacki*; 100/1.0) and the two species from northern Indochina (*P. kelomohy* and *Protobothrops* sp. from Vang Vieng, Laos; 100/1.0) form two strongly-supported, reciprocally monophyletic clades (Fig. 2).
3. Clade C (100/1.0) includes all the remaining species of the genus *Protobothrops*, which form three major subclades. The first subclade regroups two species from the Ryukyus, Japan: *P. flavoviridis* and *P. tokarensis* (100/1.0); their phylogenetic position remains unclear. The second subclade includes the wide-ranging species *P. mucrosquamatus* (100/1.0) from Indochina, India and China; *P. elegans* from the Ryukyus, Japan; and two species from southern China and northern Vietnam: *P. maolanensis* and *P. trungkhanhensis*, forming a well-supported clade (98/1.0). The second subclade regroups *P. cornutus* from Vietnam, *P. dabieshanensis* and *P. xiangchengensis* from China and a wide-ranging species, *P. jerdonii* (96/1.0). *Protobothrops cornutus* forms a sister lineage to the remaining species of this subclade (87/0.99), while *P. dabieshanensis* is reconstructed as a sister species to the *P. jerdonii* complex (100/1.0). *Protobothrops xiangchengensis* (100/1.0) is unambiguously placed within the radiation of *P. jerdonii*, making the latter species paraphyletic. The latter species includes three major lineages: a population from north-eastern India and the Himalayas (Xizang, subspecies *P. jerdonii jerdonii* Günther); populations from northern Vietnam and southern Yunnan Province of China (subspecies *P. jerdonii bourreti* Klemmer; 64/0.95); and the populations from central, western and eastern mainland China (subspecies *P. jerdonii xanthomelas* Günther; 100/1.0). Overall, the phylogenetic relationships amongst the lineages of the *P. jerdonii* species complex remain insufficiently resolved (Fig. 2).

The only substantial difference in the topology of the total evidence analysis (Fig. 2) as compared to the matrilineal genealogy (Suppl. material 1: fig. S1) is that in the mtDNA tree, Clade A was strongly (99/1.0) suggested as a sister lineage of Clade C, while Clade B occupied a more basal position within the genus in *Protobothrops*, forming a sister clade to the group including Clades A+C. In other respects, the topology of the two trees was almost identical.

Genetic distances

The uncorrected *p*-distances for the cyt *b* gene fragment amongst the *Protobothrops* species examined in the present study are presented in Suppl. material 1: appendix 5. Interspecific distances varied from $p = 1.8\%$ (between *P. tokarensis* and *P. flavoviridis*) to $p = 17.3\%$ (between *P. sieversorum* and *P. mucrosquamatus*). The newly-discovered population of *Protobothrops* sp. from Vang Vieng, Laos, differs from its sister species *P. kelomohy* by $p = 7.8\%$ and is most distantly related to *P. mucrosquamatus* ($p = 15.7\%$).

Systematics

Our molecular results strongly suggest that the newly-discovered population from Vang Vieng, Laos, indeed belongs to the genus *Protobothrops*, within which it forms a sister lineage to *P. kelomohy* from north-western Thailand (Fig. 2). Genetic divergence of the Vang Vieng *Protobothrops* sp. from its congeners approaches the species-level of divergence in the cyt *b* gene reported for many recognised species of this genus (e.g. between *P. maolanensis* and *P. elegans*, p = 7.9%; between *P. kaulbacki* and *P. himalayanus*, p = 7.9%) or even exceeds these levels for some species pairs (e.g. between *P. flavoviridis* and *P. tokarensis*, p = 1.8%; between *P. trungkhanhensis* and *P. maolanensis*, p = 5.2%; see Suppl. material 1: appendix 5). Moreover, our results indicate that the newly-discovered *Protobothrops* sp. from Vang Vieng is characterised by unique haplotypes of two nuclear DNA genes, not shared with any other *Protobothrops* species for which the homologous sequences are available (Suppl. material 1: fig. S2). Our phylogenetic results are further corroborated by the morphological comparisons (see below), which uncovered stable differences between the population of *Protobothrops* sp. from Vientiane Province, Laos and other congeners in a number of diagnostically important traits including external morphology and colouration. Based on these data and the allopatric distribution of the newly-discovered *Protobothrops* sp. (see Fig. 1) — clearly precluding any possibility of contemporary gene flow with any congener — we consider that the population *Protobothrops* sp. from Vang Vieng represents an independently evolving entity. In summary, these results support our hypothesis that the recently-discovered population of *Protobothrops* sp. from Vang Vieng, Laos, represents an unnamed species, which we formally describe below.

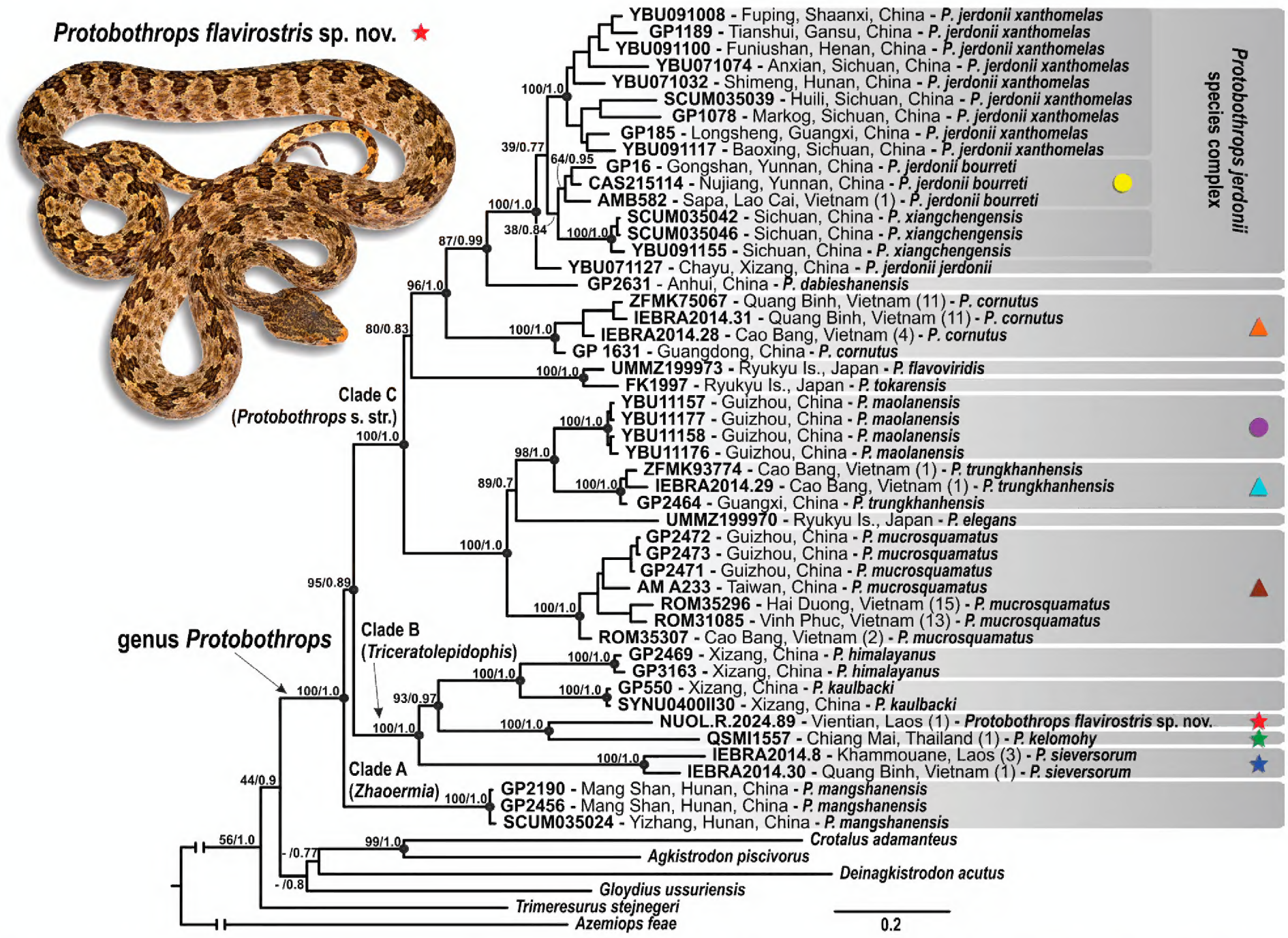
Protobothrops flavirostris sp. nov.
YBU091008 - Fuping, Shaanxi, China - P. jerdonii xanthomelas
GP1189 - Tianshui, Gansu, China - P. jerdonii xanthomelas
YBU091100 - Funiushan, Henan, China - P. jerdonii xanthomelas
YBU071074 - Anxian, Sichuan, China - P. jerdonii xanthomelas
YBU071032 - Shimeng, Hunan, China - P. jerdonii xanthomelas
SCUM035039 - Huili, Sichuan, China - P. jerdonii xanthomelas
GP1078 - Markog, Sichuan, China - P. jerdonii xanthomelas
GP185 - Longsheng, Guangxi, China - P. jerdonii xanthomelas
YBU091117 - Baoxing, Sichuan, China - P. jerdonii xanthomelas
GP16 - Gongshan, Yunnan, China - P. jerdonii bourreti
CAS215114 - Nujiang, Yunnan, China - P. jerdonii bourreti
AMB582 - Sapa, Lao Cai, Vietnam (1) - P. jerdonii bourreti
SCUM035042 - Sichuan, China - P. xiangchengensis
SCUM035046 - Sichuan, China - P. xiangchengensis
YBU091155 - Sichuan, China - P. xiangchengensis
YBU071127 - Chayu, Xizang, China - P. jerdonii jerdonii
GP2631 - Anhui, China - P. dabieshanensis
ZFMK75067 - Quang Binh, Vietnam (11) - P. cornutus
IEBRA2014.31 - Quang Binh, Vietnam (11) - P. cornutus
IEBRA2014.28 - Cao Bang, Vietnam (4) - P. cornutus
GP 1631 - Guangdong, China - P. cornutus
UMMZ199973 - Ryukyu Is., Japan - P. flavoviridis
FK1997 - Ryukyu Is., Japan - P. tokarensis
YBU11157 - Guizhou, China - P. maolanensis
YBU11177 - Guizhou, China - P. maolanensis
YBU11158 - Guizhou, China - P. maolanensis
YBU11176 - Guizhou, China - P. maolanensis
ZFMK93774 - Cao Bang, Vietnam (1) - P. trungkhanhensis
IEBRA2014.29 - Cao Bang, Vietnam (1) - P. trungkhanhensis
GP2464 - Guangxi, China - P. trungkhanhensis
UMMZ199970 - Ryukyu Is., Japan - P. elegans
GP2472 - Guizhou, China - P. mucrosquamatus
GP2473 - Guizhou, China - P. mucrosquamatus
GP2471 - Guizhou, China - P. mucrosquamatus
AM A233 - Taiwan, China - P. mucrosquamatus
ROM35296 - Hai Duong, Vietnam (15) - P. mucrosquamatus
ROM31085 - Vinh Phuc, Vietnam (13) - P. mucrosquamatus
ROM35307 - Cao Bang, Vietnam (2) - P. mucrosquamatus
GP2469 - Xizang, China - P. himalayanus
GP3163 - Xizang, China - P. himalayanus
GP550 - Xizang, China - P. kaulbacki
SYNU0400II30 - Xizang, China - P. kaulbacki
NUOL.R.2024.89 - Vientian, Laos (1) - Protobothrops flavirostris sp. nov.
QSMI1557 - Chiang Mai, Thailand (1) - P. kelomohy
IEBRA2014.8 - Khammouane, Laos (3) - P. sieversorum
IEBRA2014.30 - Quang Binh, Vietnam (1) - P. sieversorum
GP2190 - Mang Shan, Hunan, China - P. mangshanensis
GP2456 - Mang Shan, Hunan, China - P. mangshanensis
SCUM035024 - Yizhang, Hunan, China - P. mangshanensis
Crotalus adamanteus
Agkistrodon piscivorus
Deinagkistrodon acutus
Gloydius ussuriensis
Trimeresurus stejnegeri
Azemiops feae
0.2
Protobothrops jerdonii species complex
genus Protobothrops
Clade C (Protobothrops s. str.)
Clade B (Triceratolepidophis)
Clade A (Zhaoermia)
100/1.0
39/0.77
64/0.95
87/0.99
38/0.84
96/1.0
80/0.83
98/1.0
89/0.7
95/0.89
93/0.97
44/0.9
56/1.0
99/1.0
- /0.77
- /0.8

Figure 2. Maximum Likelihood (ML) phylogenetic tree of *Protobothrops* derived from the analysis of 6092 bp of cyt *b*, ND4, 12S rRNA, 16S rRNA, COI, c-mos and RAG1 gene sequences. For voucher specimen information and GenBank accession numbers, see Suppl. material 1: appendix 3. Numbers at the nodes correspond to ML UFBS/BI PP support values, respectively. Species symbols and locality numbers correspond to those provided on the map in Fig. 1. Thumbnail shows the holotype of *Protobothrops flavirostris* sp. nov. in life in dorsal view; photograph by N. Maury.

Family Viperidae Oppel, 1811
Subfamily Crotalinae Oppel, 1811
Genus *Protobothrops* Hoge & Romano-Hoge, 1983

Protobothrops flavirostris sp. nov.

https://zoobank.org/BFB5FAD4-FAD8-4F7E-89A7-C2DCC67F5E4F

Table 1, Figs 3, 4, 5B, C, Suppl. material 1: fig. S3A

Holotype. NUOL R.2024.89, adult female, from the environs of the Khan Kham Cave in Vang Vieng District, Vientiane Province, Laos (elevation 360 m a.s.l.; approximate coordinates: 18.90°N, 102.43°E; exact coordinates are not released for conservation purposes) collected by R.J. Grassby-Lewis, P. Brakels, N. Maury and S. Sitthivong on 21 October 2024.

Etymology. The specific name "*flavirostris*" is a Latin adjective in the nominative singular, masculine gender, derived from Latin words "*flāvus*" for "yellow" and "*rostrum*" for "snout" or "beak" and is given in reference to the characteristic yellowish colouration of the snout in the new species. We suggest the following common names for the new species: "Vang Vieng lance-headed pit viper" (in English), "Ngu Phao Ka Ba Khor Kop" ("ງູເຟົ້າກະບາຄໍກົບ", in Lao) and "Laosskiy habu" ("Лаосский хабу", in Russian). Furthermore, the new species is well-known to the Hmong people living in village communities around Vang Vieng and their local name for this species is "nan jaow ka," which is roughly translated as "a snake that can open its mouth very wide" or "big-mouthed snake".

Diagnosis. The new species *Protobothrops flavirostris* sp. nov. differs from other members of the genus *Protobothrops* by the following combination of the morphological characters: dorsal scales in 23–21–17 rows, all keeled; ventral scales 215; subcaudal scales 79, all paired; supralabials 7–8; infralabials 10; horn-like projection on supraocular absent; head triangular with a typical lance-shaped pattern on dorsal surface; three faint dark vertical stripes on snout; head blackish-brown with rostral, nasals, preoculars, loreals and the two anterior supralabials, as well as the anterior parts of supraoculars yellow-orange; dorsal surfaces of body and tail brown or greyish-brown, dorsum with large dark reddish-brown cross-shaped blotches, edged with black, somewhat fused together forming an interrupted zigzag line; and a row of large brown ventrolateral blotches on each side.

Description of the holotype. Adult female (Figs 3, 4), specimen in a good state of preservation. Body elongate, thin and slightly compressed (SVL 688 mm, TaL 137 mm, TL 825 mm; TaL/TL ratio 0.17) (Fig. 3A, B). Head triangular in dorsal view (Fig. 3E), elongated, clearly distinct from the neck (HL 25.4 mm, HW 16.2 mm, HD 10.2 mm; HW/HL ratio 0.64); covered with small, convex and irregularly-shaped scales (Fig. 3E). Snout elongate, dorsally flattened and rounded in dorsal view (Fig. 3E), rather rectangular in lateral view (Fig. 3C, D), with a distinct and sharp canthus rostralis (SnL 7.9 mm, IN 5.4 mm, NL 3.0 mm). Eyes medium-sized (ED 3.5 mm, EN 5.7 mm; ED/EN ratio 0.61). Rostral triangular, barely visible in dorsal aspect (Fig. 3E). Pupil vertically elliptical. Loreal pit present, large, triangular in shape, located closer to eye than to nostril (Fig. 3C, D). Nostril vertical, elliptical, completely enclosed in nasal scale, with a short complete suture dividing the ventral part of nasal just below nostril (Fig. 3C, D). Nasal scale separated from first supralabial by a complete suture (Fig. 3C, D). Subocular long, thin, crescent-like, separated from the fourth supralabial by a single scale and separated from the fifth supralabial by 2/2 scales (Fig. 3C, D). Three preoculars on each side of the head; two upper preoculars located dorsally of the loreal pit; first preocular short, rectangular; second preocular elongated, bordering the dorsal margin of loreal pit; the first upper preocular in contact with the posterior loreal; the second upper preoculars in contact with the anterior and posterior loreals, which separate it from the nasal; lower preocular triangular, elongated, forming the ventral margin of the loreal pit; lower preocular in contact with subocular, third and second supralabials (Fig. 3C, D). A small elongated, rectangular scale between nasal and second supralabial, also in contact with anterior loreal and first supralabial (Fig. 3C, D); 2/2 postoculars; 8/7 supralabials, third the largest (Fig. 3C, D); 10/10 infralabials, those of the first pair in contact with each other behind the triangular mental; the first two pairs of infralabials in contact with the single pair of chin shields (Fig. 3F). Four pairs of gulars aligned between the chin shields and the first preventral (Fig. 3F). One pair of enlarged internasals, not in contact with rostral and separated from each other by two small scales (Fig. 3E). One large supraocular, with 7–8 small scales along the transverse line between supraoculars (Fig. 3E). Scales on snout and in the interorbital region smooth, irregular, subimbricate; temporal and occipital scales feebly keeled (Fig. 3E, F). Dorsal scales in 23–21–17 rows, all of them narrow, pointed and strongly keeled in single and straight keels, including the first row. One preventral and 215 ventrals. Cloacal plate single; 79 subcaudals, all divided (Fig. 3B).

Colouration. In life (Fig. 4), the dorsum pale brown to greyish-brown with dense dark brown mottling and ca. 87/86 large dark reddish-brown cross-shaped blotches, edged in black, somewhat fused together, forming an interrupted and irregular zigzag line (Fig. 3A). Tail pale yellow above with dark brown blotches, becoming darker to blackish-brown towards the tail tip, intermittent with small irregular patches of white scales at the tail tip (Fig. 3A). Dorsal surfaces of the head pale yellow to orange-yellow with oblique dark brown stripes and blotches; the background colouration becoming more brightly yellow towards the snout (Fig. 3C, D, E). Tongue pinkish-purple. Eyes greyish-brown with dark-brown mottling forming an irregular horizontal dark ocular stripe and a bright golden-yellow edging of the pupil (Fig. 3C, D). Ventral surfaces pale brown to beige with darker brownish speckling, white blotches and dark brown polygonal spots, which become darker along the lateral sides of the venter. Ventral surfaces becoming cream to off-white on the neck and throat, with the same pattern consisting of dark brownish spots and speckles.

Table 1. Comparison of morphological characters of *Protobothrops flavirostris* sp. nov. with 15 currently recognised species of the genus *Protobothrops*. Symbol characters are: n = sampe size; (1) = ratio TaL/TL; (2) = number VEN; (3) = number SC; (4) = number ASR; (5) = number MSR; (6) = number PSR; (7) = number SL; (8) = number IL; (9) = Supraoculars (flat = 0; horns = 1); (10) = 1st DSR (smooth = 0; keeled = 1). Remarks: Diagnostic differences from the new species are marked in bold.

Species	n	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	Sources
P. flavirostris **sp. nov.**	1	0.17	215	79	23	21	17	7–8	10–10	0	1	10
P. cornutus	8	0.14–0.24	**187–202**	**65–78**	**19(21)**	21(19)	17(15)	**9–10**	10–14	**1**	**0**	5, 7
P. dabieshanensis	1	0.14–0.21	**187**	**54–58**	**21**	21	**15**	8–9	**11–12**	0	**0**	5
P. elegans	16	**0.18–0.23**	**179–192**	63–79	23–31	**23–25**	**19**	7–8	10–12	0	**0**	1, 6
P. flavoviridis	12	0.15–0.19	**220–237**	76–91	**37–41**	**33–39**	**23–25**	7–9	10–12	0	**0**	1, 6
P. himalayanus	3	0.15–0.18	198–216	**65–76**	**25**	**25**	**19**	7–8	**11–13**	0	**0**	6
P. jerdonii	30	0.15–0.17	**160–192**	44–80	21–23	21–23	15–17	7–8	**11–12**	0	**0**	2, 6
P. kaulbacki	3	0.17–0.20	**201–212**	66–86	**25**	**25–27**	17–19	7–8	**12–14**	0	**0**	2, 5
P. kelomohy	2	**0.18–0.22**	**231–234**	**80–84**	23	**23**	17	8–9	**12–13**	0	1	7
P. mangshanensis	19	0.13–0.15	**187–198**	**60–67**	**25**	**25**	17	**14–15**	**13–16**	0	**0**	2, 5
P. maolanensis	5	0.16–0.21	**186–199**	74–85	23–25	19(21)	15(17)	7–10	**11–12**	0	1	4
P. mucrosquamatus	25	0.11–0.24	193–233	78–100	**25–28**	**23–27**	17–25	**9–11**	**11–17**	0	**0**	2, 6, 9
P. sieversorum	17	0.16–0.23	**228–235**	79–82	**23–24**	21–23	17	8–10	**13–14**	**1**	1	9
P. tokarensis	12	0.15–0.18	**203–208**	74–83	**35**	**31**	**19**	7–9	**12–16**	0	**0**	1, 6
P. trungkhanhensis	2	**0.21–0.24**	**188–194**	**75–76**	**19**	**19**	17	8–9	**11–12**	0	1	3
P. xiangchengensis	8	**0.13–0.15**	**175–194**	**44–66**	**25**	**23–25**	15–17	7–8	**11–13**	0	**0**	2, 6

Sources: 1 = Maki (1931); 2 = Zhao et al. (1998); 3 = Orlov et al. (2009); 4 = Yang et al. (2011); 5 = Huang et al. (2012); 6 = Pan et al. (2013); 7 = Luu et al. (2015); 8 = Sumontha et al. (2020); 9 = David et al. (2023); 10 = this study.

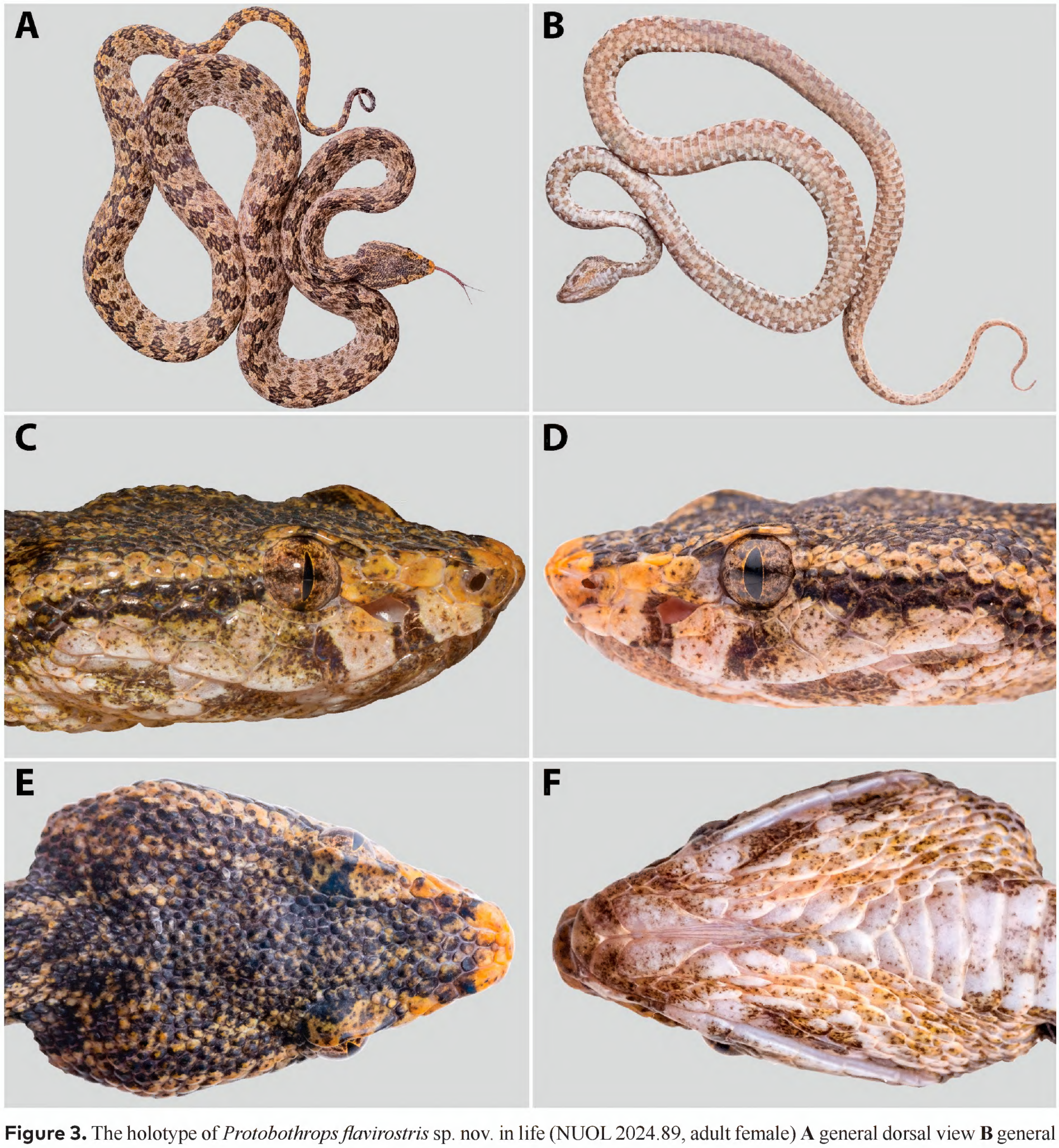

Figure 3. The holotype of *Protobothrops flavirostris* sp. nov. in life (NUOL 2024.89, adult female) **A** general dorsal view **B** general ventral view **C** lateral view of the head, right side **D** lateral view of the head, left side **E** dorsal view of the head; **F** Ventral view of the head. Photographs by R.J. Grassby-Lewis (**A, B, D–F**) and N. Maury (**C**).

After five months in preservative, the bright colours of the specimen have somewhat faded; the yellowish colouration of the snout, the tail and the eyes have completely disappeared and the general pattern on the dorsum and venter remained unchanged.

Variation. As only a single specimen was collected by us, the observations on morphological variation of the new species provided below are very scarce and are based on two photo records obtained through the citizen-science platform iNaturalist (www.inaturalist.org). Therefore, we were only able to observe some minor variation of colouration in life of two additional not-collected specimens. The new species was recorded from the Khan Kham Cave (Vang Vieng, Laos) in May 2023 (observation #159203732) and showed somewhat darker colouration with a grey base colour and no bright yellow marking on the snout (Fig. 5C); however, the quality of this photo, possibly due to insufficient lighting conditions, is moderate and it is not easy to described the details of the body colouration of this specimen. Another specimen of an unknown sex was recorded from the Nang Oua Khiam Cave (Vang Vieng, Laos) in November 2023 (observation #190687143), the colouration of this specimen of an unknown sex agreeing very well with the description of the holotype, including the brown

Figure 4. The holotype of *Protobothrops flavirostris* sp. nov. in life *in situ* (NUOL 2024.89, adult female). Photograph by R.J. Grassby-Lewis.

background colour and yellowish snout (Fig. 5B). Another uncollected and unphotographed specimen, observed by I. Stelmah at the Loup Cave in May 2024, also had more greyish rather than brownish base colouration and had a yellowish blotch on its snout (Inna Stelmah, pers. comm.). Overall, there is a certain variation in the background colouration in the new species (greyish or brownish); however, the body pattern is quite similar and the presence of yellowish colouration on the end of the tail and the snout appears to be a diagnostic feature of the new species.

Comparisons. The main diagnostic characters separating *Protobothrops flavirostris* sp. nov. from all other 15 currently recognised species of the genus *Protobothrops* are summarised in Table 1 and further illustrated in Suppl. material 1: figs S3, S4.

Protobothrops flavirostris sp. nov. is readily distinguished from *P. cornutus* (distributed in northern and central Vietnam and southernmost China) by having a higher number of ventrals (VEN 215 vs. 187–202); a higher ASR number (23 vs. 19, rarely 21); a lower number of supralabials (SL 7–8 vs. 9–10); the absence of a horn-like projection on supraocular (vs. presence); by having the outermost row of dorsal scales keeled (vs. smooth); and by having a clearly different colour pattern (see Suppl. material 1: fig. S3A vs. fig. S3B).

The new species is distinguished from *P. dabieshanensis* (endemic to central China) by having a higher VEN number (215 vs. 187); a higher SC number (79 vs. 54–58); a higher ASR number (23 vs. 21); a higher PSR number (17 vs. 15); a lower IL number (10 vs. 11–12); by having the outermost row of dorsal scales keeled (vs. smooth); and by having a markedly different colour pattern (see Suppl. material 1: fig. S3A vs. fig. S3C).

Protobothrops flavirostris sp. nov. is further distinguished from *P. elegans* (endemic to the southern Ryukyu Islands or Yaeyama Islands of Japan) by having a lower TaL/TL ratio (0.17 vs. 0.18–0.23); a lower MSR number (21 vs. 23–25); a lower PSR number (17 vs. 19); two loreals (vs. one); by having the outermost row of dorsal scales keeled (vs. smooth); and by having a different colour pattern (see Suppl. material 1: fig. S3A vs. fig. S3D).

The new species is further distinguished from *P. flavoviridis* (endemic to the Okinawa and Amami island groups of the Ryukyu Islands, Japan) by having a lower VEN number (215 vs. 220–237); a lower ASR number (23 vs. 37–41); a lower MSR number (21 vs. 33–39); a lower PSR number (17 vs. 23–25); two loreals (vs. one); by having the outermost row of dorsal scales keeled (vs. smooth); and by having a clearly different colour pattern (see Suppl. material 1: fig. S3A vs. fig. S3E).

Protobothrops flavirostris sp. nov. is further distinguished from *P. himalayanus* (distributed in the Himalayan Region, including Nepal, Bhutan, Xizang Province of China and northeast India) by having a higher SC number (79 vs. 65–76); a lower ASR number (23 vs. 25); a lower MSR number (21 vs. 25); a lower PSR number

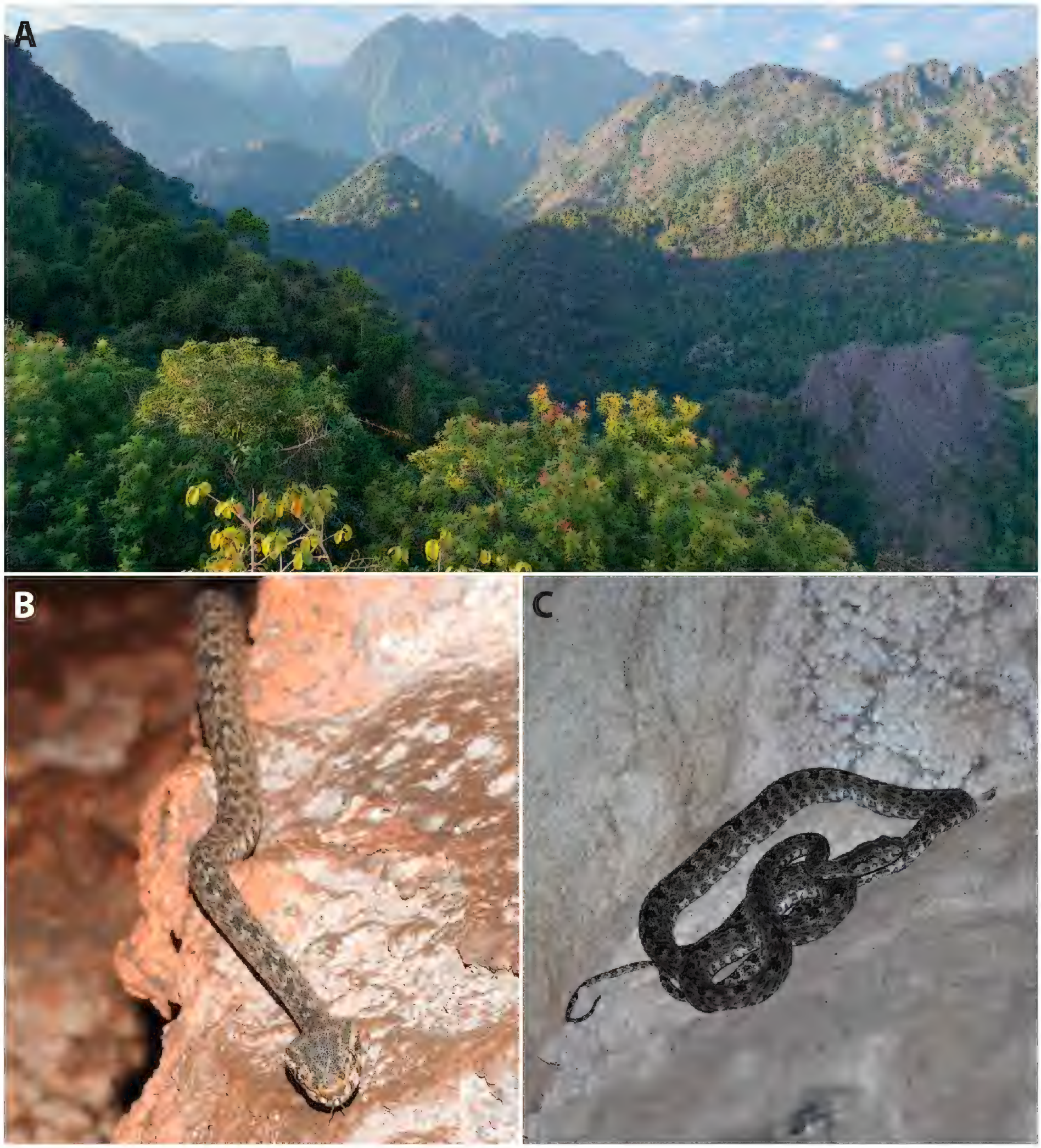

Figure 5. Habitat of *Protobothrops flavirostris* sp. nov. Macrohabitat of the new species at the type locality, Vang Vieng District, Vientiane Province, Laos (**A**); uncollected adult snake (sex unknown) from Nang Oua Khiam Cave, Vang Vieng, Vientian, Laos in life in situ (**B**); uncollected adult snake (sex unknown) from Khan Kham Cave, Vang Vieng, Vientian, Laos in life in situ (**C**). Photographs by P. Brakels (**A**); J. Wohlschiess (**B**); and P.L. Stenger (**C**).

(17 vs. 19); a lower IL number (10 vs. 11–13); by having the outermost row of dorsal scales keeled (vs. smooth); and by having a different colour pattern (see Suppl. material 1: fig. S3A vs. fig. S3F).

The new species is also easily distinguished from the members of *P. jerdonii* complex (including: *P. j. jerdonii*, *P. j. bourreti* and *P. j. xanthomelas*) distributed in Nepal, Bhutan, southern and central China, northeast India, northern Myanmar and northern Vietnam by having a higher VEN number (215 vs. 160–192); a lower IL number (10 vs. 11, 12); by having the outermost row of dorsal scales keeled (vs. smooth); and by having a markedly different colour pattern (see Suppl. material 1: fig. S3A vs. fig. S3G–I).

Protobothrops flavirostris sp. nov. is distinguished from *P. kaulbacki* (distributed in the Eastern Himalayas in northern Myanmar, south-western China and northeast India) by having a slightly higher VEN number (215 vs. 201–212); a lower ASR number (23 vs. 25); a lower MSR number (21 vs. 25–27); a lower IL number (10 vs. 12–14); by having the outermost row of dorsal scales keeled

(vs. smooth); and by having a clearly different colour pattern (see Suppl. material 1: fig. S3A vs. fig. S4A).

The new species is easily distinguished from *P. mangshanensis* (endemic to southern China) by having a higher TaL/TL ratio (0.17 vs. 0.13–0.15); a higher VEN number (215 vs. 187–198); a higher SC number (79 vs. 60–67); a lower ASR number (23 vs. 25); a lower MSR number (21 vs. 25); a lower SL number (7–8 vs. 14–15); a lower IL number (10 vs. 13–16); two loreals (vs. one); by having the outermost row of dorsal scales keeled (vs. smooth); and by having a different colour pattern (see Suppl. material 1: fig. S3A vs. fig. S4C).

Protobothrops flavirostris sp. nov. is further distinguished from *P. maolanensis* (distributed in southern China and northern Vietnam) by having a higher VEN number (215 vs. 186–199); a lower IL number (10 vs. 11–12); a higher number of dark blotches on dorsum (87 blotches vs. 68–72 cross-bands); and by having a generally different colour pattern (see Suppl. material 1: fig. S3A vs. fig. S4D).

Protobothrops flavirostris sp. nov. is easily distinguished from *P. mucrosquamatus* (distributed across northeast India, western and central China, including the Taiwan Island, northern and central Vietnam, central and southern Laos, north-eastern Thailand and also introduced to Okinawa Island of Japan) by having a lower ASR number (23 vs. 25–28); a lower MSR number (21 vs. 23–27); a lower SL number (7–8 vs. 9–11); by having the outermost row of dorsal scales keeled (vs. smooth); and by having a distinctly different colour pattern (see Suppl. material 1: fig. S3A vs. fig. S4E).

Protobothrops flavirostris sp. nov. is easily distinguished from *P. sieversorum* (endemic to the Annamite (or Truong Son) Range in central Vietnam and central Laos) by having a lower VEN number (215 vs. 228–235); a lower IL number (10 vs. 13–14); by the absence of a horn-like projection on supraocular (vs. present); and by having a generally different colour pattern (see Suppl. material 1: fig. S3A vs. fig. S4F).

The new species is further distinguished from *P. tokarensis* (endemic to the Tokara Islands within the Ryukyu Islands of Japan) by having a higher VEN number (215 vs. 203–208); a lower ASR number (23 vs. 35); a lower MSR number (21 vs. 31); a lower PSR number (17 vs. 19); a lower IL number (10 vs. 12–16); two loreals (vs. one); by having the outermost row of dorsal scales keeled (vs. smooth); and by having a different colour pattern (see Suppl. material 1: fig. S3A vs. fig. S4G).

Protobothrops flavirostris sp. nov. is easily distinguished from *P. trungkhanhensis* (distributed in north-eastern Vietnam and southern China) by having lower TaL/TL ratio (0.17 vs. 0.21–0.24); a higher VEN number (215 vs. 188–194); a slightly higher SC number (79 vs. 75–76); a higher ASR number (23 vs. 19); a higher MSR number (21 vs. 19); a lower IL number (10 vs. 11–12); and by having a different colour pattern (see Suppl. material 1: fig. S3A vs. fig. S4H).

The new species is further distinguished from *P. xiangchengensis* (endemic to south-western China) by having a higher TaL/TL ratio (0.17 vs. 0.13–0.15); a higher VEN number (215 vs. 175–194); a higher SC number (79 vs. 44–66); a lower ASR number (23 vs. 25); a lower MSR number (21 vs. 23–25); a lower IL number (10 vs. 11–13); by having the outermost row of dorsal scales keeled (vs. smooth); and by having a clearly different colour pattern (see Suppl. material 1: fig. S3A vs. fig. S4I).

Finally, *Protobothrops flavirostris* sp. nov. can be readily distinguished from its sister species *P. kelomohy* (endemic to Dawna Range in north-western Thailand) by having: a slightly lower TaL/TL ratio (0.17 vs. 0.18–0.22); a lower number of ventrals (VEN 215 vs. 231–234; a lower number of mid-body scale rows (MSR 21 vs. 23); a lower number of infralabials (IL 10 vs. 12–13); by having two loreals (vs. one); and by the following colouration differences (see Suppl. material 1: fig. S3A vs. fig. S4B for comparison). The new species has a higher number of dark blotches on the dorsum (86 or 87 vs. 63–67); dorsally a bright yellowish-orange snout and tail (vs. no yellowish colouration on the snout and tail); transverse dark stripes on the snout brownish with irregular and posteriorly indistinct edges (Fig. 3C, D) (vs. contrasting blackish transverse stripes on snout and lateral sides of the head, see Suppl. material 1: fig. S4B); and by having background colouration greyish-brown to light brown (vs. reddish-brown, see Suppl. material 1: fig. S4B).

Distribution and natural history notes. Currently, *Protobothrops flavirostris* sp. nov. is known only from three limestone caves within the Vang Vieng karstic massif (Fig. 5A), namely Khan Kham Cave, Nang Oua Khiam Cave and Loup Caves, all located within the Vang Vieng District, Vientiane Province, Laos. Though at present the species can be considered microendemic to a narrow area in Vang Vieng karsts, its occurrence in the adjacent limestone karst formations in other provinces of north-western Laos, such as Xayabury, Luang Prabang and Xaysombom Provinces, cannot be excluded. *Protobothrops flavirostris* sp. nov. was recorded at low elevations (200–400 m a.s.l.) in limestone massifs covered by karst forest or within the caves.

Protobothrops flavirostris sp. nov. is nocturnal; the holotype was collected at 21h15, although all individuals seen within caves were observed during the daytime hours. All four known observations of the new species are from within caves on limestone karst formations or at the base of limestone karst formations. All four individuals were observed climbing on limestone rocks, stalactites/stalagmites or cave walls. The holotype specimen would constantly anchor itself to anything possible using its tail and was very agile while climbing. Hereby, we identify the new species as a specialised limestone-dwelling rupicolous ecotype, spending most of its life climbing or resting amongst steep limestone rocks and hiding in their crevices.

The diet of the new species is not reliably known, though it may include the local species of bats (Chiroptera), as the species was only recorded in the caves with remnant bat colonies. Bent-toed geckos (primarily *Cyrtodactylus pageli* Schneider, Nguyen, Schmitz, Kingsada, Auer & Ziegler) were abundant within these same caves and on the adjacent limestone karst formations, so it is possible that it also predates on geckos. Other species of

amphibians and reptiles recorded at the same habitat in the type locality during our survey included *Microhyla mukhlesuri* Hasan, Islam, Kuramoto, Kurabayashi & Sumida, *Micryletta* cf. *menglienica* (Yang & Su), *Kaloula pulchra* Gray, *Sylvirana* cf. *menglaensis* (Fei, Ye & Xie), *Polypedates megacephalus* Hallowell, *Gracixalus* sp., *Rhacophorus kio* Ohler & Delorme, *Acanthosaura* cf. *rubrilabris* Liu, Rao, Hou, Orlov, Ananjeva & Zhang, *Cyrtodactylus pageli*, *Gehyra mutilata* (Wiegmann), *Ahaetulla prasina* (Boie), *Lycodon davidi* Vogel, Nguyen, Kingsada & Ziegler, *L. davisonii* (Blanford), *L. neomaculatus* Nguyen, Lee, Pauwels, Kennedy-Gold, Poyarkov, David & Vogel, *Bungarus fasciatus* (Schneider), *Trimeresurus macrops* Kramer and *T. guoi* Chen, Shi, Vogel & Ding.

Conservation status. To date, despite intensive survey efforts by our team and local herpers in the area, only four individuals of the new species have been recorded in relatively close vicinity. This indicates that *Protobothrops flavirostris* sp. nov. is likely highly secretive and elusive. Since the new species is a highly specialised limestone karst-dweller, the main threats to it are habitat loss and habitat disturbance, while it can also be subject to human prosecution from the locals due to stigma and fear of its dangerous venom. We presume this species to be restricted to limestone karst formations, which are directly threatened around Vang Vieng, mostly due to cement manufacturing, but also due to mining for ores such as iron, gold and other rare ores. Mining of these karst formations has accelerated in recent years. The suitable limestone karst habitat in Vang Vieng is not legally protected by a national park or wildlife sanctuary. If the new species indeed represents a point-endemic restricted to the karst formations surrounding Vang Vieng, this poses a significant threat to its survival. Furthermore, the discovery and formal description of this species may also lead to demand in the animal trade, which can also represent a significant threat.

In Vang Vieng District, bats are heavily sought after by local people for food, with a result that the bat population in this limestone area has been decimated. Unsustainable hunting measures are often used, such as deploying mist nets at cave entrances or exits and starting fires in the caves to flush the bats. Fire on long wooden torches that can reach the cave ceiling are used to burn bat wings so they drop to the ground and are collected for food. Bats are also commonly sold on the local market. In several independent interviews, the locals confirmed that virtually all bat populations around Vang Vieng District have been eradicated due to uncontrolled hunting and collection practices along with exploitation of the caves for tourism, which includes building infrastructure and permanent lighting in caves. This causes a severe disturbance and increases pressure on the cave ecosystems. According to the interview with the local Hmong people, *Protobothrops flavirostris* sp. nov. was much more common even 10–15 years ago, but became a very rarely observed species during the recent years.

Given the information provided above, we suggest that *Protobothrops flavirostris* sp. nov. should be considered as Endangered (EN) following the IUCN's Red List categories (IUCN Standards and Petitions Committee 2019).

Discussion

The present study focuses on the description of a new microendemic species from the genus *Protobothrops*. Furthermore, our study presents the most complete phylogeny for the genus *Protobothrops* in terms of taxon sampling published to date, which includes 16 nominal species of the genus. Overall, our phylogenetic results agree with the earlier multilocus phylogeny of *Protobothrops* presented by Guo et al. (2016) in recognising three major clades within the genus: Clade A, which includes *P. mangshanensis*; Clade B, which includes two species from the Himalayas (*P. himalayanus* and *P. kaulbacki*) and Indochina (*P. sieversorum*, *P. kelomohy* and *Protobothrops flavirostris* sp. nov.); and Clade C, which includes all the remaining species of the genus from Indochina and East Asia (Fig. 2).

The phylogenetic relationships amongst the three major *Protobothrops* clades are insufficiently resolved in our total evidence analysis tree with Clades B and C reconstructed as sister clades with moderate levels of node support (95/0.89; see Fig. 2). This topology contradicts the results of Guo et al. (2016), in which Clade A is strongly suggested as a sister group of Clade C in the BI analysis, but not the ML analysis (87/1.0). According to our matrilineal genealogy of the genus *Protobothrops* (Suppl. material 1: fig. S1), Clades A+C are strongly supported as a monophyletic group (99/1.0) with Clade B forming a sister group to the latter. Though the study of Guo et al. (2016) included four mtDNA (cyt b, ND4, 12S rRNA and 16S rRNA) and four nDNA genes (c-mos, RAG1, PRLR and UBN1), while our phylogeny is based on the analyses of five mtDNA and two nDNA genes (see above), it seems that these data are insufficient to fully resolve the basal nodes of *Protobothrops* radiation. It is likely that the topology B+(A+C) mostly results from the mtDNA phylogenetic signal, but is not supported by the nuDNA genes. Unfortunately, a recent multilocus study of Chinese snakes by Li et al. (2020) did not include *P. mangshanensis* (Clade A) nor any species of Clade B in their analysis. Therefore, further multilocus studies involving larger gene and taxon sampling are required to clarify the topology of phylogenetic relationships in the genus *Protobothrops*.

The taxonomic status of the three main clades of *Protobothrops* also requires further studies. *Protobothrops sieversorum* was originally described as a member of the monotypic genus *Triceratolepidophis*, based on a number of unique morphological character states found in this species. *Protobothrops mangshanensis* was originally described as a member of the genus *Trimeresurus*, but was later placed in a monotypic genus *Ermia* by Zhang (1993), based on significant differences in skull morphology. However, as the nomen for the genus *Ermia* was already in use for a genus of locusts and was preoccupied,

Gumprecht and Tillack (2004) provided a replacement name for this taxon and the species was assigned to a monotypic genus *Zhaoermia*. The first molecular phylogenetic data on these snakes, provided by Guo et al. (2007), indicated close relationships between *Zhaoermia* and *Triceratolepidophis* and *Protobothrops* and both former genera were coined as subjective junior synonyms of *Protobothrops* (Guo et al. 2007). *Protobothrops mangshanensis* is one of the largest members of the genus and is clearly different from other congeners in both external morphology, colouration and skull morphology (see Zhang (1993)). Though the overall morphology of *Protobothrops sieversorum* appears to be greatly different from the other members of Clade B (including the new species *Protobothrops flavirostris* sp. nov.), a comprehensive examination of external and osteological characters of various *Protobothrops* species is still lacking. Therefore, as all molecular studies on the group agree that the monophyly of the genus *Protobothrops* sensu lato is unambiguously supported and it includes three major deeply-divergent clades, we think that they may well be regarded as subgenera: *Zhaoermia* stat. nov. (for Clade A), *Triceratolepidophis* stat. nov. (for Clade B) and *Protobothrops* stat. nov. (for Clade C) (for the phylogenetic definition of each of the proposed subgenera and the included species, see Suppl. material 1: appendix 7). Further thorough morphological and osteological studies are required to examine morphological variation in these groups; they might reveal additional diagnostic characters for each of the putative subgenera of the genus *Protobothrops*.

Despite the significant progress made in recent years in understanding the diversity and distribution of the genus *Protobothrops*, the taxonomy of the group still has some unresolved issues. In particular, the taxonomy of the *P. jerdonii* species complex remains incomplete. A number of previous works (Guo et al. 2006, 2009, 2016) demonstrated that *P. xiangchengensis* is closely related to the wide-ranging polytypic species, *P. jerdonii*. In particular, Guo et al. (2006) provided the first phylogenetic data on *P. xiangchengensis* and demonstrated that it is placed within the radiation of *P. jerdonii*, rendering the latter species paraphyletic. Paradoxically enough, the authors stated that their results "confirm that *P. xiangchengensis* is a valid species"; however, they refrained from a proper revision of the *P. jerdonii* species complex. Subsequent phylogenetic studies, including Guo et al. (2016) and Li et al. (2020), only included a small subset or a single sample of *P. jerdonii* in their analyses, so as a result, *P. jerdonii* and *P. xiangchengensis* would look as sister species on the phylogenetic tree, which is actually not the case (see Fig. 2). The degree of divergence between *P. jerdonii* and *P. xiangchengensis* in cyt *b* gene sequences is also quite low and is notably smaller than that of most other recognised species of *Protobothrops* (p = 4.0%) (see Suppl. material 1: appendix 5). Though we recognise that *P. xiangchengensis* represents a unique phylogenetic lineage which is both molecularly and morphologically distinct from its congeners and, thus, warrants formal taxonomic recognition, given the phylogenetic placement of this taxon within the radition of *P. jerdonii*, it should be either considered a subspecies of *P. jerdonii* or the taxonomy of the whole *P. jerdonii* complex should be further revised and the currently recognised subspecies *P. jerdonii jerdonii*, *P. jerdonii bourreti* and *P. jerdonii xanthomelas* should be raised in rank to full species. The latter act would require a detailed study of morphological variation in the *P. jerdonii* complex across its vast range, accompanied by a multilocus phylogenetic study of its genetic differentiation. At present, all available data on the phylogenetic differentiation of the *P. jerdonii* complex and *P. xiangchengensis* are based entirely on mtDNA gene sequences and it is widely accepted now that matrilineal genealogy alone cannot be used as a basis for taxonomic decisions (see Dufresnes et al. (2023); Wüster et al. (2024)). In the present study, we refrain from making any taxonomic decisions on the status of *P. xiangchengensis*, but we would like to underline that the validity of this species requires further confirmation by multilocus phylogenetic analysis and a thorough assessment of morphological differentiation.

Although the authors conducted several field surveys in Vang Vieng District from 2021 to 2024, the presumed rarity of *Protobothrops flavirostris* sp. nov., its cryptic camouflage (slender body and greyish-brown colour) and secretive ecology made it difficult to detect in the field, resulting in only one specimen being collected and examined. Its sister species, *P. kelomohy*, is similarly a rare and very elusive species, with a low number of specimens recorded and collected (Sumontha et al. 2020) despite significant survey efforts to its range over many years. At the type locality, *Protobothrops flavirostris* sp. nov. was observed twice in May (1 May 2024 and 10 May 2024) and twice in autumn (21 October 2024 and 1 November 2024). Though our data are insufficient, this may indicate that the new species has seasonal changes in its activity and the snakes might be more active at the beginning and at the end of the rainy season. This assumption is consistent with reports from local Hmong villagers, who reported that they primarily see the new species at the end of the rainy season, from late October to mid-November.

While the type specimen of *Protobothrops flavirostris* sp. nov. was found climbing amongst limestone rocks at the base of a karst formation with no cave system nearby, all three photo records obtained through citizen science were observed *in situ* within cave systems. It is still unclear if the new species is a cave-dwelling specialist or not. The authors intensively surveyed caves (both remote unvisited and popular tourist caves) on many occasions through different seasons, without observing a single individual. Vang Vieng is one of the most popular locations in Southeast Asia for spelunking, with hundreds of tourists visiting caves in this area every day. This could have resulted in a significant bias in the survey, as individuals of *Protobothrops flavirostris* sp. nov. that spontaneously entered caves to cool off or search for bats during the day were more likely to be recorded. Local Hmong villagers who were

asked about this species of snake also reported that they have observed them along limestone karst streams perching on rocks and very rarely inside caves when questioned. Therefore, we should consider *Protobothrops flavirostris* sp. nov. a limestone karst-dwelling species that may opportunistically enter caves perhaps for feeding, but is likely not a primarily cave-dwelling troglophilous species. Similar ecology was also recently reported in a number of Southeast Asian snake species (e.g. Grismer et al. (2014); Idiiatullina et al. (2023); Pawangkhanant et al. (2024)).

We would like to underline that *Protobothrops flavirostris* sp. nov. appears to be an endangered species. Of course, its actual range may be larger than currently known and the species may be recorded in other limestone massifs along the Luang Prabang Range in western Laos. The unsustainable hunting measures, including fires created by locals to flush bats into their mist nets and burn their wings, artificial lighting in popular tourist caves and high tourist traffic, have created significant disturbance to all the accessible caves in the Vang Vieng area. We assume that the new species is endemic to a range-restricted area similar to other *Protobothrops* species directly associated with limestone massifs, such as *P. maolanensis*, *P. trungkhanhensis* and *P. sieversorum*. We strongly recommend additional field surveys, especially in the limestone areas of the Luang Prabang Range, including those in the Provinces of Vientiane (Mueang Feuang and Kasi Districts), Xayabury, Luang Prabang (or Luangphrabang) and Xaysombom. The discovery of additional populations of *Protobothrops flavirostris* sp. nov. is crucial for an accurate estimation of its conservation status, as well as for obtaining data on the ecology and behaviour of this new species. Toxicology of the new species also remains unknown, though, according to the interviews with local people, its bite is deemed dangerous for humans and can be life threatening.

In terms of herpetofaunal diversity, Laos has been a critically undersurveyed country for many years (Poyarkov et al. 2021, 2023). There is a defined lack of records of *Protobothrops* species in both northern and southern parts of the country (see Fig. 1), which is most likely explained by the lack of survey efforts in Laos. In recent years, increasing numbers of field surveys have led to advancements in Laotian herpetofauna assessments and resulted in a rapid increase in the number of recorded amphibian and reptile species. This is especially due to surveys in limestone karst areas, which have led to new country records and new species descriptions being published almost every year (e.g. Teynié and David (2014); Nguyen et al. (2020); Poyarkov et al. (2021, 2023); Brakels et al. (2022); Sithivong et al. (2023)). The discovery of *Protobothrops flavirostris* sp. nov., a microendemic limestone karst-specialised species of snakes, is yet another prominent addition to the herpetofauna of Laos. This discovery further underlines the need for additional herpetological studies in Laos, especially in karstic regions where there is high potential for the discovery of unique and micro-endemic species.

The discovery of *Protobothrops flavirostris* sp. nov. increases the number of *Protobothrops* species known from Laos to three and raises the total number of species of the genus *Protobothrops* to 16 (but note that the taxonomic status of *P. xiangchengensis* requires further investigation as mentioned above). Despite the recent progress in molecular taxonomy, several species complexes (e.g. the *P. jerdonii* complex, see Guo et al. (2009, 2011)) or widely distributed species (e.g. *P. mucrosquamatus*, see Guo et al. (2019)) of the lance-headed pit vipers still remain insufficiently studied. Therefore, we call for further research on the taxonomy, distribution and toxicology of the genus *Protobothrops*, especially for integrative taxonomic analyses of controversial species complexes.

Acknowledgements

We owe special thanks to the Laos Department of Forestry of the Ministry of Agriculture and Forestry of the Lao PDR, the CITES Management Division, the National University of Laos, Lao PDR and the Department of Agriculture and Forestry of Vientiane Province for their permission to conduct the field survey. Tissue sample exportation was permitted by the decision No. 0138/DoF of 09 January 2025 by the Department of Forestry of the Ministry of Agriculture and Forestry of the Lao PDR. We are especially thankful to Gernot Vogel (Germany) for sharing information related to *Protobothrops* spp. We thank Jasmin Wohlschiess, Pierre-Louis Stenge, Gernot Vogel, Yu Hao Xu, Loi Phuoc Tran, Tai Cao Kim, Ton Smith, Kenji Ito, Jun Jie Huang, Anurag Mishra, Zhong Wen Jiang and Inna Stelmah for sharing information and providing us with photographs of *Protobothrops* spp. P. Brakels expresses thanks to Kherth Chanthalard and Ketsakan Sotoukee for support during the field surveys. We also warmly thank Ngoc Quynh Nguyen and Duc Trong Nguyen (SIFASV, Vietnam) for the tremendous help in the preparation of the certain figures. We thank Patrick David, Wolfgang Wüster and Montri Sumontha for helpful comments that allowed us to improve the previous version of the manuscript. The study was conducted under the state assignmen of Lomonosov Moscow State University; this work was supported by the Russian Science Foundation to N.A. Poyarkov (Grant No. 22-14-00037, molecular and morphological analyses, data analysis) and in part by the Rufford Foundation to T.V. Nguyen (Grant No. 45888-2, morphological analyses, data analysis).

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Supplementary material 1

Supplementary information

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Link: https://doi.org/10.3897/herpetozoa.38.e146004.suppl1